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Hypoxia up-regulates angiopoietin-2, a Tie-2 ligand, in mouse mesangial cells

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Background. Angiopoietins are secreted factors modulating endothelial survival and morphogenesis. Our previous studies demonstrated angiopoietin-2 (Ang-2) promoter activity in vivo in maturing kidney vascular smooth muscle and mesangial cells, with Tie-2 expressed by adjacent endothelia, including glomerular capillaries.

Methods. In this study we investigated Ang-2 expression in immortalized mouse mesangial cell lines and studied the response to hypoxia.

Results. Using reverse transcription-polymerase chain reaction, Ang-2 and Ang-3 mRNA were detected but Ang-1 and Tie-2 transcripts were absent. As assessed by Northern and slot blotting, 8 to 24 hours hypoxia (3% O_2) significantly increased Ang-2 mRNA levels versus normoxic (21% O_2) cells and the rate of Ang-2 mRNA degradation was similar in both conditions, consistent with increased transcription. Hypoxia also increased immunoreactive Ang-2 in cell lysates. Hypoxic stimulation of Ang-2 mRNA was significantly reduced by inhibitors of tyrosine kinase (genistein) and protein kinase C (GF109203X), but not by a mitogen-activated protein kinase 1 inhibitor (PD98059). Furthermore, hypoxia coincidentally up-regulated levels of vascular endothelial growth factor (VEGF) mRNA in these cells. Finally, in vivo, immunoreactive Ang-2 was observed in the cores of immature glomeruli of neonatal mice, but immunostaining in this location was absent in four-week postnatal mice.

Conclusion. This is the first demonstration that isolated mesangial cells express Ang-2 mRNA and protein and up-regulate Ang-2 in response to hypoxia. We speculate that hypoxia-induced, mesangial-derived Ang-2 and VEGF may have synergistic paracrine roles in the growth of glomerular endothelia during normal development and diseases.

Tie (tyrosine kinase containing immunoglobulin-like loops and epidermal growth factor similar domains) genes are receptor tyrosine kinases controlling endothelial cell (EC) survival and morphogenesis [1]. As assessed by transplantation of Tie-1/LacZ metanephroi, Tie-1 expressing precursors that are present at the inception of mouse nephrogenesis can differentiate into glomerular capillaries [2], and null mutant EC in Tie-1^{lcz}/Tie-1^{lczn-} chimeric mice fail to contribute to renal vessels [3]. It has also been reported that hypoxia up-regulates the Tie-1 promoter in mouse metanephric organ culture [4]. No ligand has been reported for Tie-1.

The EC lineage also expresses Tie-2, a Tie-1 homologue [5]. Angiopoietin-1 (Ang-1) is secreted from cells near endothelia and binds Tie-2; subsequent tyrosine phosphorylation transduces EC survival and sprouting signals [6–9]. Ang-1 and Tie-2 null mutant mouse embryos have abnormal vascular networks with growth-retarded vascular smooth muscle precursors [1, 10]. Hence, Tie-2 activation also causes reciprocal, maturational effects on adjacent smooth muscle precursors, probably elicited by endothelial-derived factors such as platelet-derived growth factor-B.

Angiopoietin-2, another member of the ligand family, binds Tie-2 without causing tyrosine phosphorylation in cultured EC [11]. Instead, it antagonizes Ang-1-induced Tie-2 phosphorylation, while Ang-2 overexpression in vivo causes defects resembling Tie-2 and Ang-1 null mutants. In the presence of abundant vascular endothelial growth factor (VEGF), Ang-2 destabilizes vascular networks and facilitates sprouting, such as in tumor growth [12, 13]. Conversely, with low ambient VEGF levels, Ang-2 causes vessel regression, for example, in corpus luteum involution [11]. Ang-2 is expressed by smooth muscle cells [11, 14, 15] and also by some EC [14–16] and epithelia [15, 17]. Recently, a third member of ligand family, Ang-3, was cloned in mice: it is postulated to have a similar action as Ang-2 [18].

This study provides evidence for hypoxic up-regulation of Ang-2, in concert with VEGF, in cultured mouse kidney mesangial cells (MC) [19]. These cells act as a support for glomerular endothelia and are related to the pericyte/smooth muscle lineage. We also show that

Key words: angiopoietin, hypoxia, mesangium, endothelial cells, nephrogenesis, kidney development.

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Ang-2 protein is present, as assessed by immunostaining, in developing neonatal glomeruli.

METHODS

Reagents were obtained from Sigma Chemical Company unless otherwise specified. We used two immortal MC lines (G2 and G3) derived from six-week-old heterozygous H-2K^b-tsA58 mice [19]. They express temperature-sensitive simian virus 40 T antigen induced by low levels of interferon-γ (IFN-γ) as well as typical cultured MC markers [alpha-smooth muscle actin (α-SMA), desmin and myosin], but lack EC and epithelial markers [19]. To expand MC, cells were cultured in Dulbecco's modified Eagle's medium, 5% fetal calf serum and 40 U/mL recombinant murine IFN-γ at 33°C in a 21% O_2/5% CO_2/air atmosphere (normoxia). They were passaged into medium lacking IFN-γ and grown to semiconfluency (10^4/cm^2) when serum was withdrawn for 24 hours: this point defined the start of all experiments (0 hour). Cells were subsequently cultured in serum-free and IFN-γ-free medium in either normoxia or in a continuously-monitored atmosphere of 3% O_2/5% CO_2/92% N_2 (hypoxia) for up to 24 hours [4].

In some experiments, MC were cultured with inhibitors of tyrosine kinase (TK) (genistein; 40 μmol/L), protein kinase C (PKC; GF109203X; 5 μmol/L) or mitogen-activated protein kinase kinase 1 (MEK-1; PD98059; 25 μmol/L), as used by Oh et al. when investigating bovine EC Ang-2 expression [16]. Dimethyl sulfoxide (DMSO) was used as a solvent for stocks of inhibitors and hence appropriate vehicle-only control experiments were also performed. Degradation of RNA was measured as described [16]: after 24 hours hypoxia, actinomycin D (10 μg/mL) was added to inhibit RNA synthesis and MC were further cultured in normoxia or hypoxia for up to 7.5 hours.

RNA was extracted from MC and embryonic day 17 mouse metanephroi as described [17, 19]. Primers for reverse transcription polymerase chain reaction (RT-PCR) were based on mouse cDNA sequences: Ang-1, 5′-GGG-AGG-AAA-AAG-AGA-AGA-AGA-G (sense primer corresponding to nucleotides (nt) 1125-1146 and 5′-TGA-AAT-CAG-CAC-CGT-GTA-AG (antisense primer, nt 1563-1582) [Genbank accession number (GA) U83509] [6]; Ang-2, 5′-GGG-GAG-AAG-AGA-AGA-GAA-GAG (sense primer, nt 38-58 and 5′-CAG-GGC-ATT-GGA-CAT-TAG (antisense primer, nt 387-405) (GA AF004326) [11]; Ang-3, 5′-ACG-CTC-TCA-GCA-GCA-ATT-C (sense primer, nt 752-770) and 5′-TTC-CCA-GTC-ATG-CAG-TTC-C (antisense primer, nt 1158-1176) (GA AF113707) [18]; Tie-2, 5′-TTG-AAG-TGA-CGA-ATG-AGA-T (sense primer, nt 1601-1619) and 5′-ATT-TAG-AGC-TGT-CTG-GCT-T (antisense primer, nt 1779-1797) (GA X67553 S40311) [5].

Polymerase chain reaction (PCR) was performed for 35 cycles: one minute denaturing at 95°C, 30 seconds annealing at 60°C and 30 seconds extension at 72°C. Amplified bands were visualized after electrophoresis through ethidium bromide-stained agarose gels, and were excised and digested with appropriate restriction enzymes generating fragments consistent with reported cDNA sequences (data not shown). For negative controls, reverse transcriptase was omitted.

Northern and slot blotting for Ang-2 and 18S rRNA were performed essentially as described [17] and Ang-2 mRNA/18S rRNA ratios from three experiments were compared with one-way ANOVA, with $P < 0.05$ considered significant. Similar protocols for Northern and slot blotting were employed to detect and quantify VEGF transcripts, using a probe corresponding to base pairs 7-396 of VEGF cDNA prepared by RT-PCR of RNA from mouse metanephroi: the identity of the probe has previously been confirmed by sequencing [2].

For Ang-2 immunohistochemistry, we examined kidneys from CD1 wild-type mice. Neonatal and four-week postnatal organs were fixed in 4% paraformaldehyde and 5 μm paraffin sections were treated with proteinase for 10 minutes at room temperature. Endogenous peroxidase was quenched with 3% H_2O_2 in methanol for 30 minutes at room temperature and sections were blocked in 10% goat serum with 0.1% Tween 20. They were reacted with goat anti-human Ang-2 antibody (1:500; Santa Cruz, CA, USA). According to the data sheet from the supplier, this antibody reacts with both human and mouse Ang-2 and is not cross-reactive with Ang-1. Bound antibodies were detected with a streptavidin-biotin peroxidase system (ABC kit; DAKO, High Wycombe, UK). Negative controls included omission of the primary antibody and pre-incubation of the Ang-2 antibody with Ang-2 peptide, as described by the supplier (Santa Cruz). Sections were counterstained with hematoxylin.

For Ang-2 immunoprecipitation and Western blotting, G3 cells were cultured under standard conditions in 10 cm Petri dishes until they were nearly confluent, cultured with serum-free medium for 24 hours, and then exposed to normoxia or hypoxia for 24 hours. Cells were trypsinized and lysed in RIPA buffer. Supernatants were collected after sonication and centrifuged at 13,000 rpm for 30 minutes. Ten milligrams of protein from each condition was incubated with 10 μL of Ang-2 antibody for two hours at 4°C and 30 μL of Protein A/G agarose (Santa Cruz). Beads were washed with RIPA buffer four times and collected by centrifugation at 2500 rpm for five minutes at 4°C. After the final wash, supernatant was aspirated and discarded, and the pellet resuspended in 30 μL of 1× electrophoresis buffer. Samples were boiled for five minutes and separated on a SDS-9% PAGE gel. Proteins were transferred to nitrocellulose

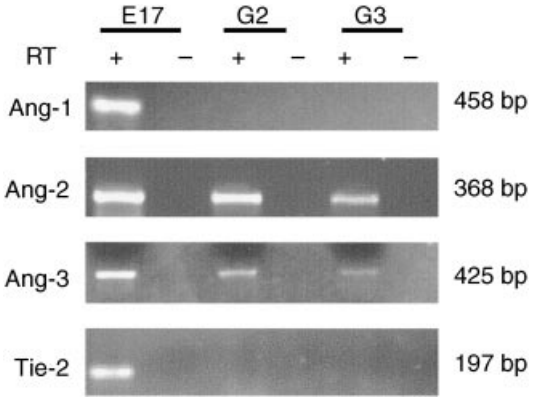

Fig. 1. Reverse transriptase-polymerase chain reaction (RT-PCR). A representative result from two experiments is shown. As assessed by RT-PCR, the embryonic day 17 metanephros (E17) from wild-type mice expressed angiopoietin-1 (Ang-1), Ang-2, Ang-3 and Tie-2. Mesangial cell (MC) lines (G2 and G3) expressed Ang-2 and Ang-3 mRNA but not Ang-1 or Tie-2. No band was amplified when reverse transcriptase was omitted. RT+ indicates experiments with reverse transcriptase; RT− indicates experiments without reverse transcriptase. Sizes of amplified bands in base pairs were: Ang-1, 458; Ang-2, 368; Ang-3, 425; Tie-2, 197.

membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) by electroblotting (Biorad, Hemel Hempstead, UK). Blots were blocked overnight at 4°C with 5% wt/vol fat free milk powder, 0.3% (vol/vol) Tween 20 in PBS and then incubated with goat anti-human Ang-2 antibody (1:5000 in blocking solution) for two hours at 4°C. After washing in blocking solution, blots were incubated for 30 minutes with horseradish peroxidase-conjugated second antibody diluted to 1:3000 in blocking solution. Blots were washed three times with blocking solution and once with PBS. Immunocomplexes were developed using an enhanced horseradish peroxidase/luminol chemiluminescence reagent (Du Pont-New England Nuclear, Boston, MA, USA). Proteins were sized with Rainbow markers. Recombinant Ang-2 (a gift from Dr. G. Yancopoulos, Regeneron Pharmaceuticals, New York, NY, USA) was used as a positive control.

RESULTS

As assessed by RT-PCR, lines G2 and G3 expressed Ang-2 and Ang-3, but not Ang-1 or Tie-2 (Fig. 1). Transcripts for Ang-1, Ang-2, Ang-3 and Tie-2 were detectable in metanephroi. Since results from both MC lines were similar, we hereafter describe G3 data only.

Angiopoietin-2 mRNA (2.8 kb) was detected in MC by Northern blotting (Fig. 2A). In hypoxia, MC Ang-2 mRNA rose incrementally at 4, 8 and 24 hours. Figure 2B demonstrates that, versus time-matched normoxic control cells, hypoxia significantly increased the Ang-2 mRNA/18S rRNA ratio at 8 ($P < 0.05$) and 24 ($P < 0.01$) hours. At 24 hours, the mean ratio in hypoxia was over twice the value in normoxia. After administration of actinomycin D to MC exposed to hypoxia for 24 hours, the decline in Ang-2 mRNA levels was not significantly different in the subsequent 7.5 hours of hypoxia versus normoxia (Fig. 2C). In cells cultured in normoxia for 24 hours, Ang-2 immunoreactive protein was detected in cell lysates by immunoprecipitation and Western blotting with a major band at 65 kD, as reported in EC [16]. The level of Ang-2 protein was increased in time-matched cells after exposure to hypoxia for 24 hours (Fig. 2D).

Hypoxic stimulation of Ang-2 mRNA was significantly reduced by inhibitors of TK ($P < 0.01$) and PKC ($P < 0.01$), but not by the MEK-1 inhibitor (Fig. 3 A, B). As assessed by Northern and slot blotting, VEGF mRNA levels were modestly but significantly increased by exposure to hypoxia for 4, 8 and 24 hours versus time-matched normoxic cells (Fig. 4 A, B). The maximal mean increase over controls occurred at four hours.

Finally, to test whether MC express Ang-2 protein in vivo, we performed immunohistochemistry for Ang-2 at two stages: in neonatal mice kidneys, when glomeruli are still being generated, and in four week post-natal kidneys, when glomeruli have matured. We found immunoreactivity in the core of maturing glomeruli, where mesangial cells reside, at a stage when capillary loops were not fully developed (Fig. 5A). In contrast, glomerular immunoreactivity was not detected using this methodology in the mature cortex (data not shown). We also detected Ang-2 immunoreactivity in the walls of cortical arterioles (Fig. 5B). As demonstrated in Figure 5 C and D, immunostaining was specific, being abolished by reabsorbing the antibody with immunizing peptide.

DISCUSSION

We previously reported that Ang-1, Ang-2 and Tie-2 are up-regulated during mouse kidney development, with levels peaking at 3 weeks after birth: subsequently these genes are down-regulated with low levels of mRNA persisting into adulthood [17]. Tie-2 is expressed by developing glomerular capillaries and medullary vasa rectae, while Ang-1 transcripts localize to nephrogenic mesenchyme, diverse tubule epithelia and maturing glomerular cells. Although the highest levels of Ang-2 transcripts occur in a subset of thin descending limbs of loops of Henle surrounding vasa rectae [15, 17], analysis of Ang-2/LacZ kidneys further revealed Ang-2 promoter activity in differentiating vessel walls and in cores of maturing glomeruli where MC reside [15]. Since MC share biosynthetic and structural properties with vascular smooth muscle cells, Ang-2 expression is consistent with this relationship. The novel immunohistochemical data from the current study support the conclusions from the Ang-2/LacZ transgene study, that is, Ang-2 protein was detected in neonatal mice in the core of developing glomeruli, where mesangial cells reside, and in the walls of cortical arterioles. In the mature kidney cortex, however,

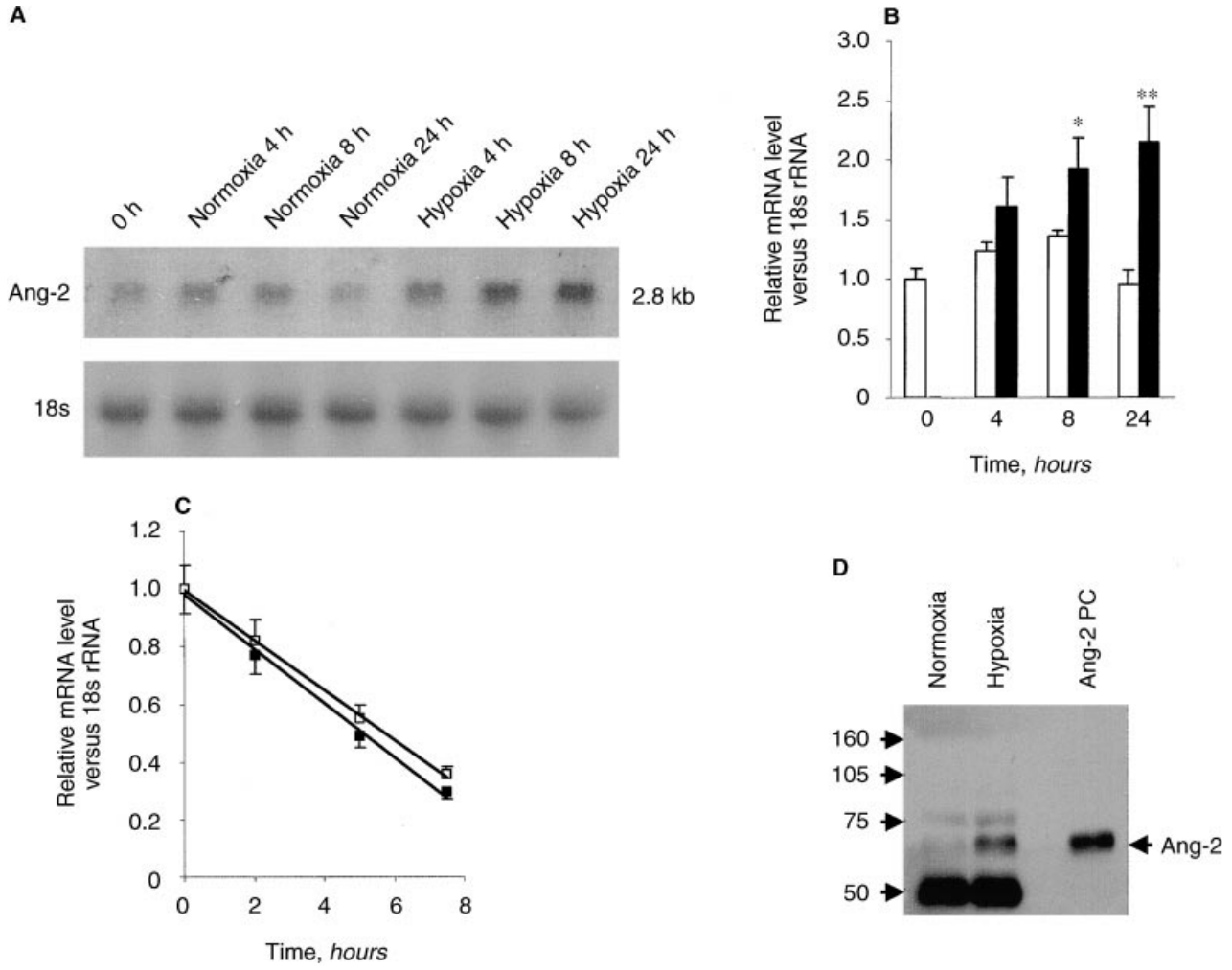

Fig. 2. Ang-2 expression in response to hypoxia. (*A*) A representative Northern blot from three experiments. MC expressed Ang-2 mRNA (2.8 kb) 24 hours after serum withdrawal (0 hr). In normoxia there was a small rise in Ang-2 mRNA at 4 and 8 hours and a fall to baseline by 24 hours. In contrast, levels of Ang-2 mRNA in hypoxia rose throughout this period. The lower half of the panel shows Northern blotting for 18S rRNA demonstrating equality of RNA loading. (*B*) Slot blotting. When compared with time-matched normoxic cells, hypoxic MC ($N = 3$) exhibited an increased Ang-2 mRNA/18S rRNA ratio that became significant at 8 hours ($^*P < 0.05$) and highly significant ($^{**}P < 0.01$) at 24 hours. (*C*) Ang-2 degradation after actinomycin D. The decline in Ang-2 mRNA/18S rRNA ($N = 3$) was not significantly different in normoxia (□) versus hypoxia (■). Means ± SD are depicted in B and C. For simplicity, in B the mean Ang-2 mRNA/18S rRNA ratio was designated '1.0' at 0 hours and in C the ratio was designated '1.0' after 24 hours hypoxia. (*D*) Ang-2 immunoprecipitation and Western blotting of 10 mg protein from MC lysates. One of two representative experiments is depicted. In MC cultured for 24 hours in normoxia, a low level of Ang-2 immunoreactive protein (around 65 kD) was detected. The intensity of this band increased when MC were exposed to hypoxia for 24 hours. Recombinant Ang-2 was included as a positive control in the far right lane. The bands at 50 kD represent unspecific signal from immunoglobulin used in the immunoprecipitation.

glomerular immunostaining could no longer be detected using our current methods.

The current study provides the first evidence that isolated renal MC express Ang-2 transcripts and secrete protein. As assessed by RT-PCR, MC also expressed Ang-3, but not Ang-1 or Tie-2 mRNA, although we cannot exclude the presence of extremely low levels of the latter two transcripts. Angiopoietins secreted by MC would be well-placed to elicit paracrine growth modulation of glomerular EC, which express Tie-2 mRNA and protein during maturation [17]. Interestingly, cultured MC also secrete other EC growth factors including hepatocyte growth factor and VEGF [19, 20].

Hypoxia is known to affect MC proliferation, matrix production and secretion of signaling molecules [21–23]. Our data further demonstrate that MC regulate Ang-2 in response to hypoxia. Since Ang-2 mRNA degradation was similar in normoxia and hypoxia, it is likely that hypoxia enhances Ang-2 gene transcription; however, proof of this hypothesis would require nuclear run-on transcription analyses. Hypoxia has also been reported to up-regulate Ang-2 mRNA in cultured bovine EC by increasing transcription [14, 16].

Hypoxia has been reported to activate PKC in rat MC [21]. Furthermore, VEGF-induced up-regulation of Ang-2 in bovine EC was abolished by TK or MEK-1

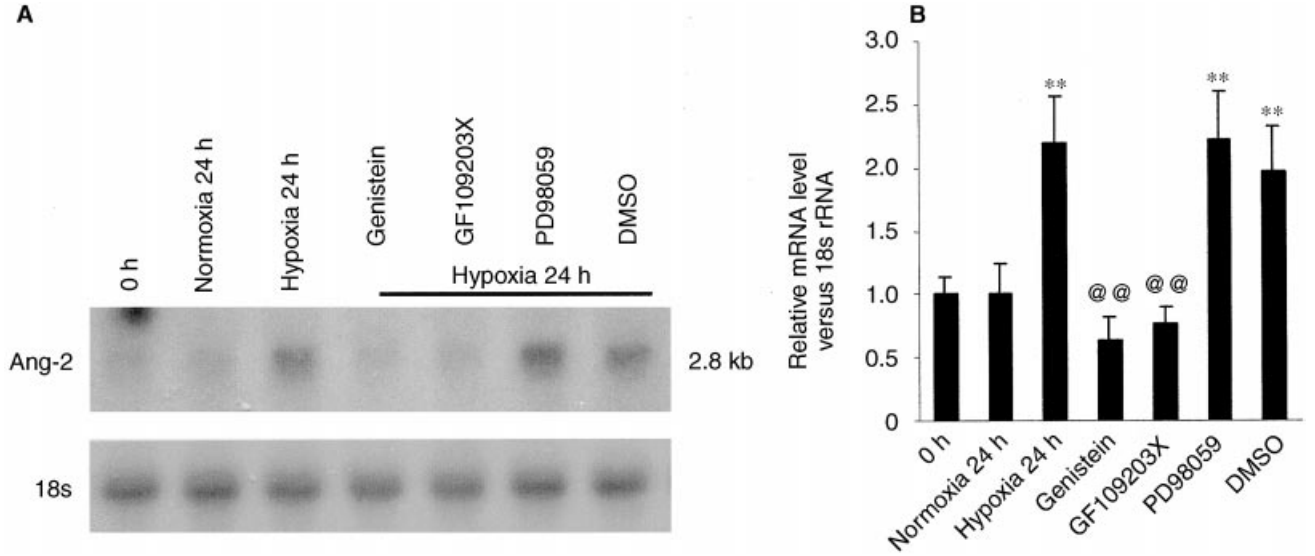

Fig. 3. Effects of metabolic inhibitors on Ang-2 mRNA levels. (*A*) Representative Northern blot from three experiments of MC harvested at 0 hours, 24 hours normoxia, 24 hours hypoxia, 24 hours of hypoxia with each of the three inhibitors and at 24 hours hypoxia with DMSO vehicle only. Hypoxic stimulation of Ang-2 mRNA was reduced by inhibitors of TK (genistein) and PKC (GF109203X) but not by the MEK-1 inhibitor (PD98059). The lower half of the panel shows Northern blotting for 18S rRNA demonstrating equality of loading. (*B*) Slot blotting. MC ($N = 3$) cultured in hypoxia in standard media (hypoxia 24 hr), in hypoxia with MEK-1 inhibitor (PD98059) or in hypoxia with vehicle alone (DMSO) showed similar significant (**$P < 0.01$) increases of Ang-2 mRNA/18S RNA ratios versus time-matched normoxic control cells (normoxia 24 hr). Compared with time-matched hypoxic cells (hypoxia 24 hr), TK (genistein) and PKC (GF109203X) inhibitors significantly (@@$P < 0.01$) reduced Ang-2 mRNA/18S rRNA ratios. In B, means ± SD are depicted.

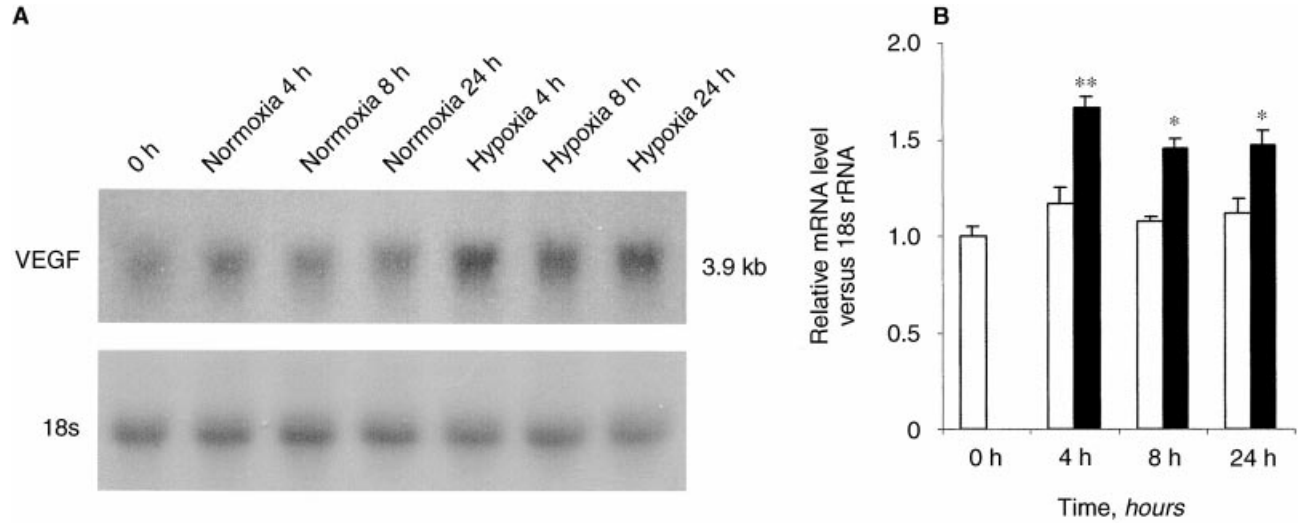

Fig. 4. Northern and slot blotting for VEGF. (*A*) Representative Northern blot from three experiments. MC expressed VEGF mRNA (3.9 kb) 24 hours after serum withdrawal (0 hr). In normoxia levels were unchanged between 0 and 24 hours whereas hypoxia induced higher levels of mRNA. The lower half of the panel shows Northern blotting for 18S rRNA demonstrating equality of RNA loading. (*B*) Slot blotting. When compared with time-matched normoxic cells, hypoxic MC ($N = 3$) exhibited a modest but significantly increased VEGF mRNA/18S rRNA ratio at 4, 8 and 24 hours versus time-matched normoxic controls (*$P < 0.05$; **$P < 0.01$).

inhibitors and partially by PKC inhibition [16]. In comparison, we found that hypoxic stimulation of Ang-2 mRNA in MC was prevented by TK and PKC inhibitors, but not by the MEK-1 inhibitor. The lack of effect of MEK-1 inhibition was not an artifact, because we have found this same maneuver inhibits other types of MC responses (H.T.Y. and A.S.W., unpublished observations). Hence, the postulated effect of hypoxia on *Ang-2* gene transcription is independent of MEK-1. Further work is required to define signaling pathways [24].

These effects on Ang-2 expression by MC should be taken in the wider context of hypoxic up-regulation of

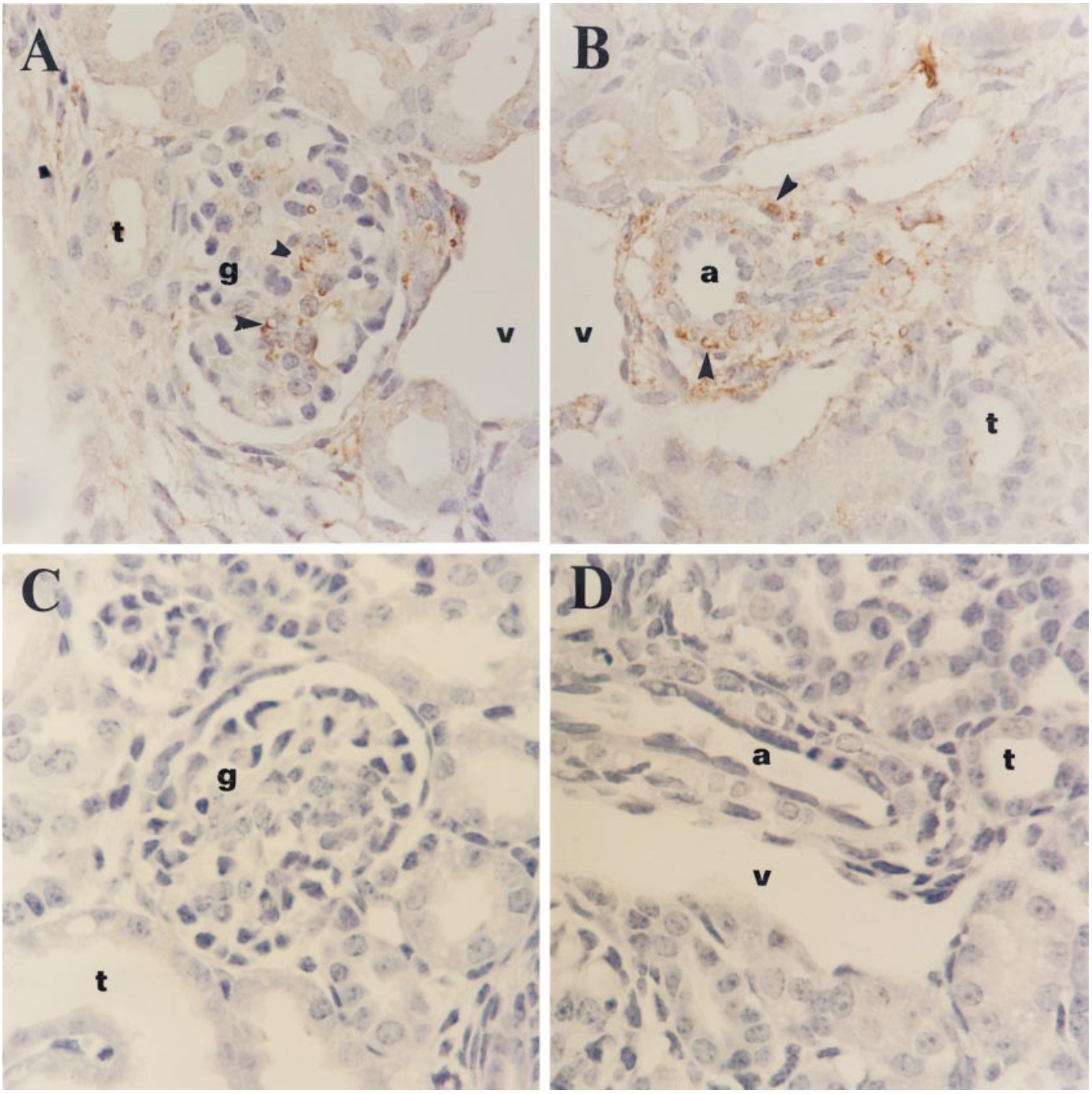

Fig. 5. Immunohistochemistry for Ang-2. Histology from the cortex of neonatal mice were counterstained with hematoxylin. *A* and *B* were immunostained with an Ang-2 antibody while *C* and *D* were complimentary negative control sections reacted with antibody reabsorbed with immunizing peptide. Positive immunoreactivity is brown and was detected in the cores of incompletely vascularized glomeruli (arrowheads in A), where mesangial cells reside, and in layers of cells around arterioles (arrowheads in B). All frames are ×63 objective. Abbreviations are: a, arteriole; g, glomerulus; t, tubules; v, vein.

diverse genes that determine EC growth: Tie-1 and Tie-2 by enhanced transcription [4, 25]; VEGF by enhanced transcription and mRNA stability [26]; VEGF receptors 1 and 2, respectively, by increased transcription [27] and VEGF paracrine stimulation [28]. It has previously been established that human MC express VEGF [20], and we confirmed this observation in our mouse MC lines. In the current study we further established that the hypoxia modestly but significantly up-regulates VEGF mRNA in these cells, with levels peaking at four hours and declining thereafter. Hence, it is reasonable to conclude that hypoxia has the potential to elicit MC expression

of both Ang-2 and VEGF in vivo. In future studies, the potentially complex bioactivities of hypoxic MC conditioned medium will need to be assessed in vitro by addition to Tie-2 and VEGF receptor expressing EC.

We think that hypoxic Ang-2 up-regulation may have three important in vivo correlates. First, during normal development glomeruli might be relatively ischemic/hypoxic because of incompletely developed capillaries. Here, Ang-2 secreted from differentiating MC [15] would enhance EC survival and sprouting in concert with VEGF and Ang-1, factors known to be expressed by maturing glomerular epithelia [17, 29]. This contention is partly supported by our current finding that glomerular Ang-2 immunoreactive protein is limited to maturing structures, which have incompletely developed capillary loops (Fig. 5A). Second, in postnatal diseases where glomeruli are rendered ischemic/hypoxic from capillary obliteration or destruction, MC-derived Ang-2 may enhance EC regeneration in synergy with VEGF [30]. Finally, it is notable that the major site of Ang-2 expression in the healthy adult kidney is a subset of tubules in the outer medulla proximale to vasa rectae [15, 17]. Here, epithelial-derived Ang-2 may have a yet to be understood role in maintaining the structural integrity of the microcirculation. We speculate that the perpetual hypoxia, which is known to occur in the medulla, may tonically up-regulate Ang-2 in this locality.

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